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Nigella sativa Modulates Cytokines Expression in Mature Bovine Adipocytes

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Abstract: In this study, we examined the effect of either lipopolysaccharide (LPS, 1 µg mL^{-1}), *Nigella.sativa* extract (NS, 5 µg mL^{-1}), or co-treatment of both for 24 h on cytokines expression in mature bovine adipocytes using RT-PCR analysis. The results showed that separate treatment by LPS and NS stimulated the expression of interleukin-1 (IL-1β), IL-6, IL-8 and IL-10. Co-treatment of cells by *N. sativa* with LPS inhibited LPS induced IL-6 and TNF-α expression and induced additive stimulatory effect on LPS induced IL-8 and IL-10 expression. The results indicate that *N. sativa* extract has immuno-modulatory effect on bovine adipocytes by stimulating different cytokines expression that potentiate different inflammatory and anti-inflammatory functions in bovine adipocytes.

Key words: Adipocytes, cytokines expression, LPS, *Nigella sativa*

INTRODUCTION

The seeds of *Nigella sativa* (NS) which are commonly known as black seed or black cumin are used in herbal medicine all over the world for treatment and prevention of a number of diseases and conditions that include asthma, hypertension, diabetes, inflammation, cough, bronchitis, headache, eczema, fever, dizziness and influenza (Salem, 2005). The seeds or its oil are used as a carminative, diuretic, lactagogue and vermifuge. *Nigella sativa* is used in food as a spice and a condiment (Ali and Blunden, 2003). NS has an antitussive effect and induces codeine like effect (Boskabady *et al.*, 2004). It contains more than 30% fixed oil and 0.40-0.45% w/w volatile oil. The volatile oil has been shown to contain 18-24% thymoquinone and 46% comprises many monoterpenes such as p-cymene and (Pinene 3) (EL-Tahir *et al.*, 1993). *Nigella sativa* was recently subjected to extensive pharmacological investigations that revealed its antioxidant activity in different organs and acted as anticancer drugs (Houghton *et al.*, 1995; Worthen *et al.*, 1998). However, the effects of NS extract on bovine adipocytes function and alteration are still unknown.

Cytokines are relatively low-molecular-weight proteins produced by many cell types (Feghali and Wright, 1997). They are pharmacologically active, exhibiting both beneficial and pathologic effects on the target cells. Imbalanced expression of cytokines has been implicated in the progression of many diseases (Arend and Gabay, 2004). TNF-α has been shown to enhance anti-tumor responses and to promote tumor regression (Peron *et al.*, 1999). IL-6 is a cytokine produced by a number of normal and transformed cells (Bartold and Haynes, 1991) and promotes or inhibits the growth of tumor cells depending upon the cell type (Feghali and Wright, 1997).

White adipose tissue secrets different cytokines that are important in modulating body defensive mechanisms such as IL-1, IL-6, TNF, IL-10 and IL-8. Adipose tissue also secrets various proteins as leptin, adiponectin and haptoglobin which are involved in regulation of different body conditions (Havel, 2004).

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Most of already established findings focused on the effect of NS on cytokines production from lymphocytes and peripheral blood mononuclear cells (El-Obeid *et al.*, 2005). For our knowledge, there are no data available about the effect of NS on bovine adipocytes and that is the purpose of this study.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagles's Medium (DMEM), Bovine Serum Albumin (BSA) and Hank's balanced salt solution were bought from Sigma-Aldrich Fine Chemical (St. Louis, MO, USA). Fetal Calf Serum (FCS) was from Trace Scientific Ltd. (Melbourne, Australia), LPS and collagenase were purchased from Wako Pure Chemicals Co. (Osaka, Japan).

Isolation of Mature Adipocytes

The experimental procedure and care of animals were in accordance with the guidelines of the Animal Care and Use Committee of Benha University. Subcutaneous adipose tissue (S/C AT) was obtained from three non-pregnant and non-lactating healthy Holstein cows (4 years old). (S/C AT) was dissected into small pieces and immersed in Hank's balanced salt solution containing 2 mg mL^{-1} collagenase and 0.1% BSA in sterile 50 mL plastic tube for digestion. After digestion at 37°C for 90 min with gentle shaking, the solution was filtered through sterile nylon mesh with 80 µm pores. The filtrate was centrifuged at 1000×g for 5 min at room temperature. The supernatant cells (mature adipocytes) were washed twice with DMEM containing 10% FCS, 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin. The cells were counted by haemocytometer and the percentage of live to dead cells was encountered by tryban blue staining. 5×10^6 cells were used for each milliliter media. The cells were cultured in DMEM (2.5 mM glucose) containing 10% FCS 6-well plates under different experimental conditions for 24 h.

Preparation of *Nigella sativa* Extract

As described before by Ibrahim *et al.* (2008), *N. sativa* seeds were purchased from the local Egyptian market. The seeds were washed, dried and crushed to a powder with an electric microniser. 20 g of the powder was dissolved in 200 mL^{-1} phosphate buffer saline (pH 6.4) and centrifuged at 10000 rpm for 30 min at 4°C to remove residual solid debris. The clear supernatant was collected as the soluble extract after removal of the oily layer and insoluble pellet. Protein concentration was determined by Lowry *et al.* (1951) method.

Experimental Procedures and RT-PCR Analysis

Cells were incubated with PBS as control or LPS (µg mL^{-1}) and/or with NS extract (5 µg mL^{-1}). The dose of both LPS and NS was determined after several preliminary traits and it was the optimal sublethal dose. Cells were incubated at 37°C in 5% CO$_2$ for 24 h (optimal time to induce different gene expression in cattle adipocytes). Total cellular RNA was isolated from cultured adipocytes by the guanidine-isothiocyanate method using TRIzol reagent (Gibco BRL, Rochville, MD, USA). RNA (1 µg) was treated at 72°C for 5 min and reverse transcribed using 100 units of Moloney murine leukemia virus reverse transcriptase (Gibco), 50 pmol of poly (dT) primer and 20 nmol of dNTPs in a total volume of 10 µL at 37°C for 1 h. After heating at 94°C for 5 min, PCR amplification was performed with 2.5 units Taq polymerase (Perkin-Elmer, Foster City, CA, USA), 3 mM MgCl$_2$ and 50 pmol of forward and reverse primers specific for respective genes in a total volume of 50 µL. The PCR conditions for different tested genes are shown in Table 1. The experiments were repeated 3 times on mature adipocytes of 3 cows. After electrophoresis in 1.5% agarose gel, the PCR products were stained with ethidium bromide and visualized under UV lamp.

Table 1: PCR primers

mRNA expression	Forward primer	Reverse primer	Treatments
GAPDH (452 bp)	5'- ACCACTGTCCACGCCATCAC-3'	5'- TCCACCACCCTGTTTGCTGTA-3'	Annealing at 59°C for 30 sec for 25 cycles
TNF-α (590 bp)	5'- TAACAAGCCGGTAGCCCACG-3'	5'- GCAAGGGCTCTTGATGGCAGA-3'	Annealing at 94°C for 30 sec for 30 cycles
IL-8 (308 bp)	5'-ATGACTTCCAAACTGGCTGTTGC-3'	5'- TCATGGATCTTGCTTCTCAGCTC-3	Annealing at 58°C for 1 min for 30 cycles
IL-1β (726 bp)	5'-ATGGCAACCGTACCTGAACCCA-3'	5'-GCTCGAAAATGTCCCAGGAA-3'	Annealing at 60°C for 1 min for 28 cycles
IL-10 (416 bp)	5'- CTACTCTGTTGCCTGGTCTTCCT-3'	5'- CTTCACCTTCTCCACCGCCTTGC-3'	Annealing at 61°C 1 min for 30 cycles
IL-6 (624 bp)	5'- ATGAACTCCCGCTTCACAAG-3'	5'-CTACTTCATCCGAATAGCTCTCA-3'	Annealing at 58°C for 1 min for 30 cycles

In the parenthesis, amplification and size of PCR product are shown. Annealing temperature, time and number of PCR cycle of respective genes are also shown, while temperature and time of denaturation and elongation steps of each PCR cycle are 94°C, 30 sec and 72°C, 60 sec, respectively

Statistical Analysis

All data were expressed as Means±SEM and analyzed by Dunnett's t-test for multiple comparisons with a single control group using Stat View program. Significance was reported as $p < 0.05$.

RESULTS

Effect of NS Extract on IL-6, TNF-'' andIL-1$ mRNA Expression

In Fig. 1 and 2, treatment of bovine adipocytes with LPS up-regulated significantly ($p < 0.05$) mRNA expression of TNF-α and IL-6. LPS is known to be the potent stimulator of different cytokines during acute phase inflammation and/or diseases. NS extract up-regulated IL-6 mRNA expression but did not affect TNF-α expression. Co-treatment of NS extract and LPS more potently down-regulated mRNA expression of TNF-α and IL-6 than that induced by LPS treatment alone.

Regarding IL-1 β expression, as shown in Fig. 3, LPS induced 2 fold increase ($p < 0.05$) in IL-1β expression while treatment of cells with NS extract induced IL-1β expression (about 1.8 fold increase) but co-treatment of LPS with NS extract did not show any changes in IL-1β expression than that induced by either LPS or NS extract although very small additive stimulatory effect is seen but not significant. These findings clearly and collectively show the role of NS extract in inhibiting the proinflammatory mediators, which are known as acute phase cytokines.

Effect of NS Extract on IL-8 and IL-10 mRNA Expression

IL-8 is a cytokine produced by adipocytes, monocytes, neutrophils and NK cells and acts as a chemoattractant factor for neutrophils, basophils and T-cells. Treatment of mature adipocytes by LPS did not affect IL-8 mRNA expression as shown in Fig. 4 but NS extract stimulated IL-8 mRNA expression. Combination of both LPS and NS extract partially induced additive effect on IL-8 expression. IL-10 is anti-inflammatory cytokine that inhibits pro-inflammatory cytokines. On the same line, treatment of bovine adipocytes by LPS (Fig. 5), induced 6 fold increase in IL-10 mRNA expression ($p < 0.05$). NS extract treatment slightly induced IL-10 expression (1 fold increase). Combination of both LPS and NS extract showed an additive up-regulation in mRNA expression of IL-10. Those findings clarify the role of *N. Sativa* as chemoattractant and regenerative factor to potentiate different body defensive mechanisms.
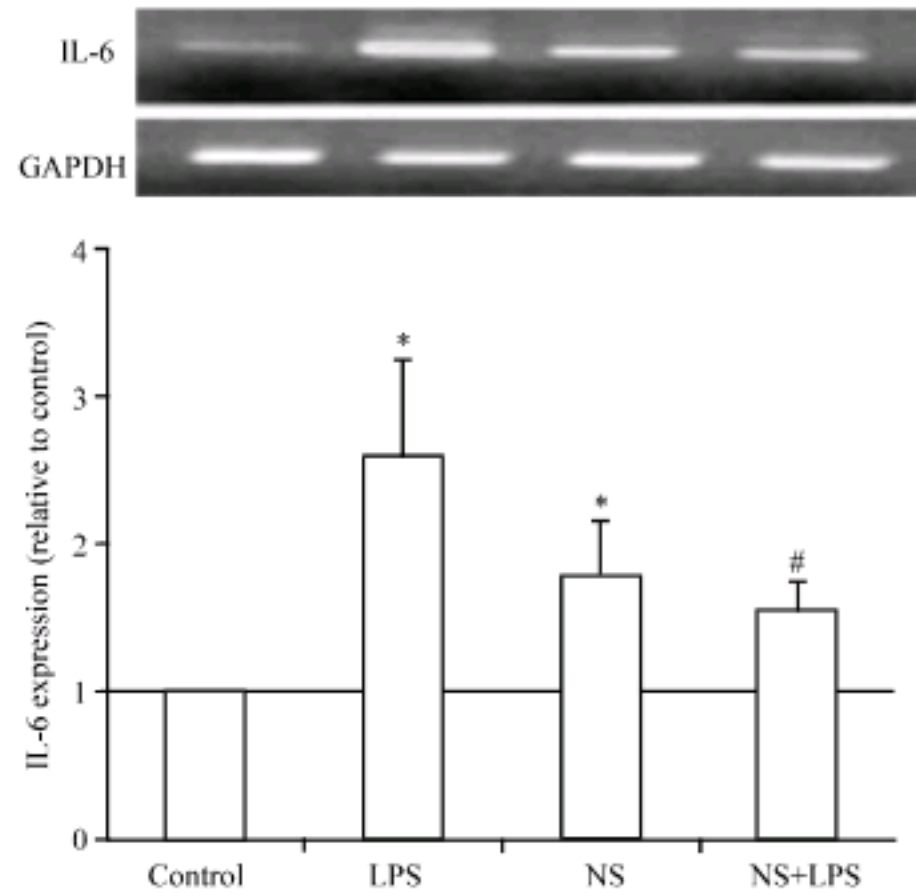
Fig. 1: Effect of *Nigella sativa* on LPS induced IL-6 expression in bovine adipocytes. Mature bovine adipocytes were cultured in 6-well plated at 5×10^6 per well with or without 5 µg mL^{-1} in presence or absence of LPS (1 µg mL^{-1}). Two microgram RNA was extracted and reverse transcribed using RT-PCR analysis for IL-6 expression. The bands of both genes were visualized using 1.5% gel stained with ethedium bromide. Intensity of expression was normalized with that of GAPDH (as internal standard) using NIH image for densitometric analysis. Values are expressed as Mean±SEM obtained from 3 experiments. *$p < 0.05$ vs. control and #$p < 0.05$ vs. LPS
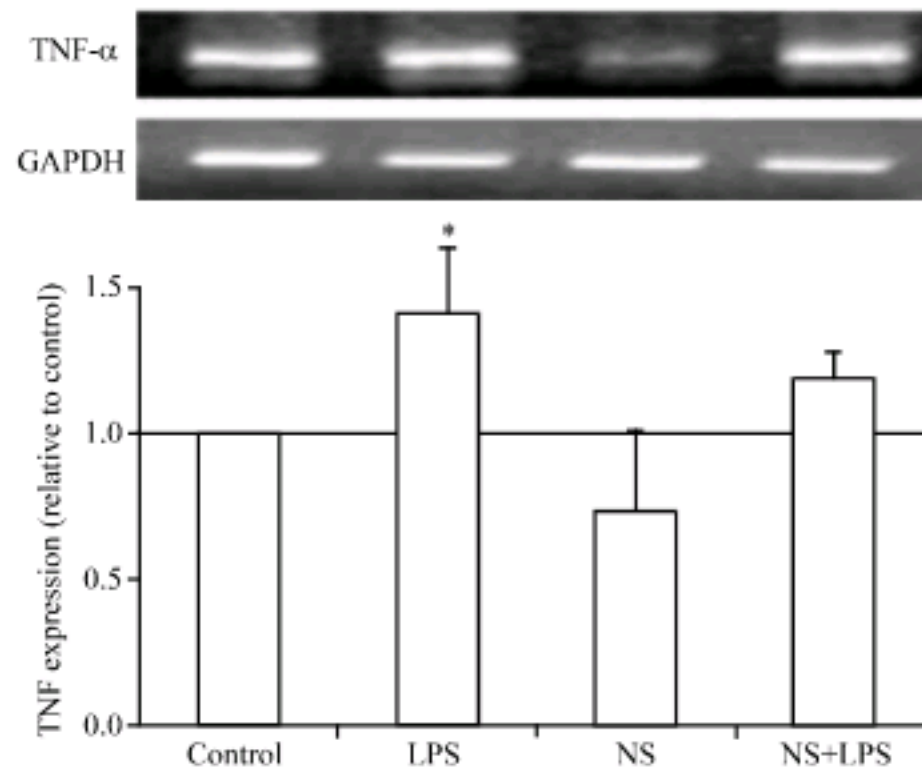

Fig. 2: Effect of *N. sativa* on LPS induced TNF-α expression in bovine adipocytes. Mature bovine adipocytes were cultured as described in Fig. 1. Two microgram RNA was reverse transcribed and subjected to RT-PCR analysis using specific primers for TNF-α and GAPDH as internal standard. Values are expressed as Mean±SEM obtained from 3 experiments. *$p < 0.05$ vs. control
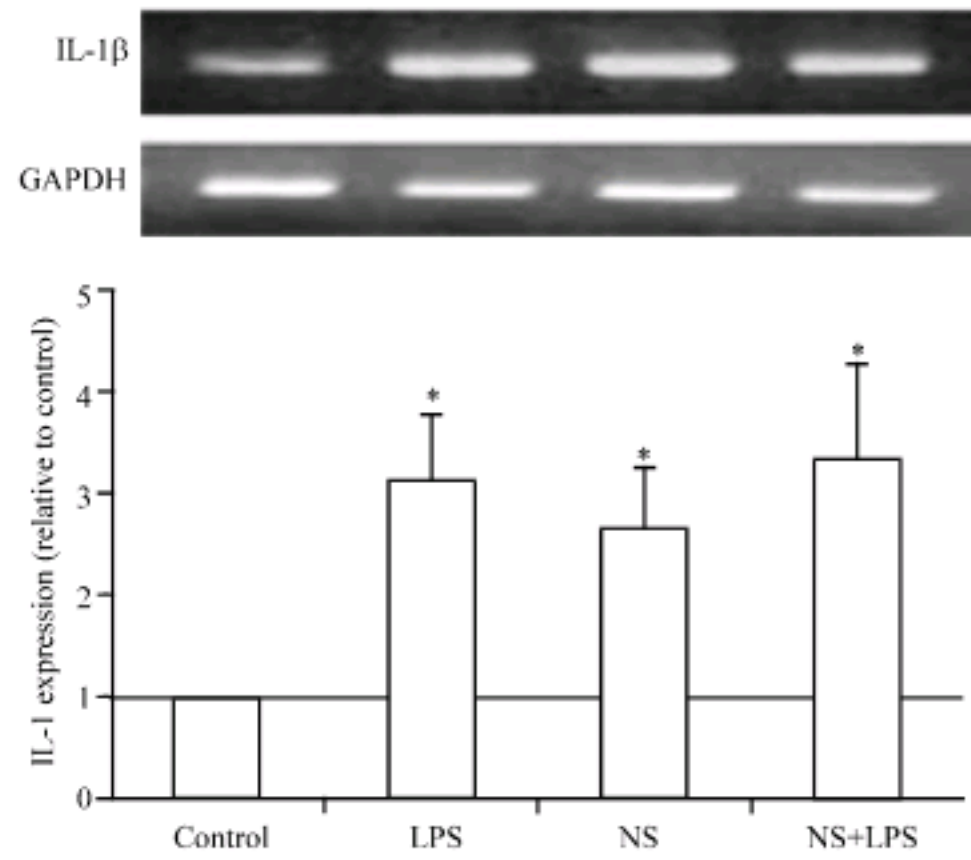
Fig. 3: Effect of *N. sativa* on LPS induced IL-1-β expression in bovine adipocytes. Mature bovine adipocytes were cultured as described in Fig. 1. Two microgram RNA was reverse transcribed and subjected to RT-PCR analysis using specific primers for IL1-β and GAPDH as internal standard. Values are expressed as Mean±SEM obtained from 3 experiments. *p<0.05 vs. control
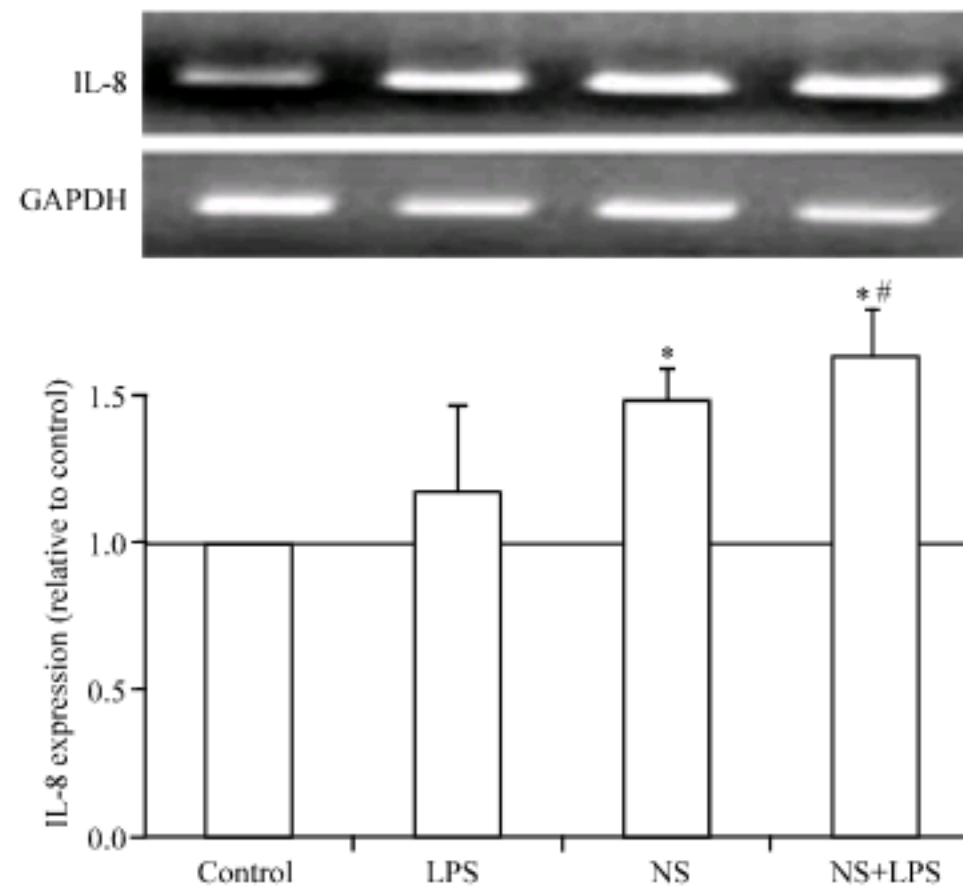

Fig. 4: Effect of *N. sativa* on LPS induced IL-8 expression in bovine adipocytes. Mature bovine adipocytes were cultured as described in Fig. 1. Two microgram RNA was reverse transcribed and subjected to RT-PCR analysis using specific primers for IL-8 and GAPDH as internal standard. Values are expressed as Mean±SEM obtained from 3 experiments. *p<0.05 vs. control and #p<0.05 vs. LPS
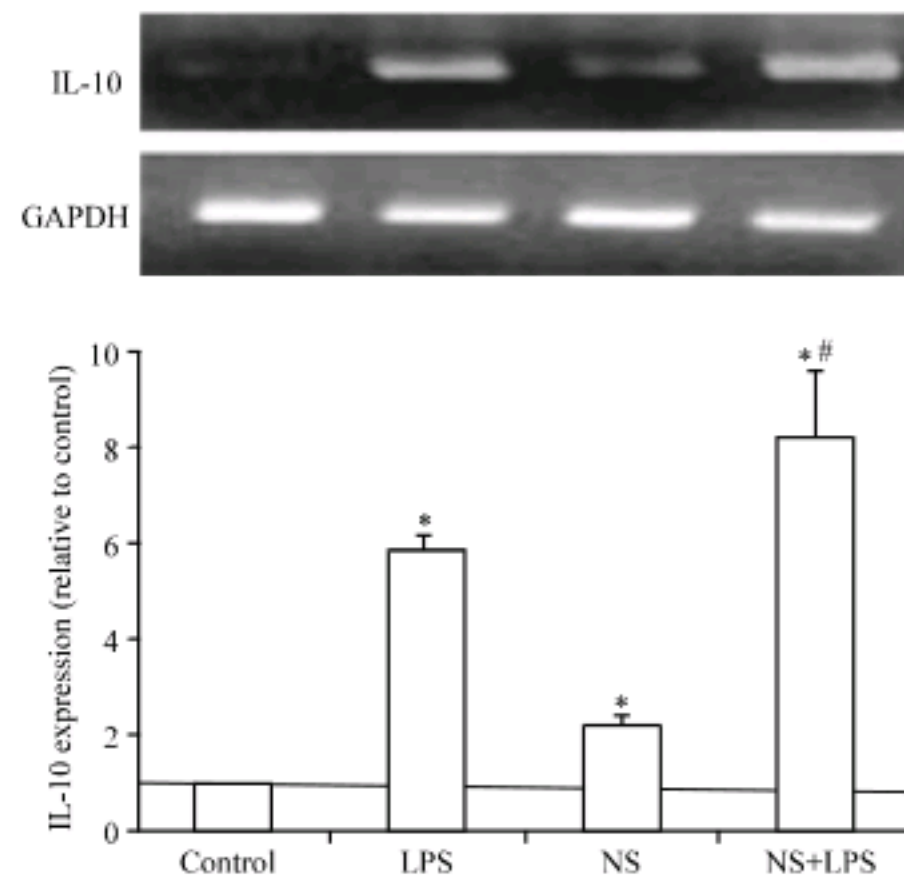
Fig. 5: Effect of *N. sativa* on LPS induced IL-10 expression in bovine adipocytes. Mature bovine adipocytes were cultured as described in Fig. 1. Two microgram RNA was reverse transcribed and subjected to RT-PCR analysis using specific primers for IL-10 and GAPDH as internal standard. Values are expressed as Mean±SEM obtained from 3 experiments. *p<0.05 vs. control and #p<0.05 vs. LPS

DISCUSSION

The present findings showed the immuno-modulatory effect of *N. sativa* extract on LPS induced different inflammatory cytokines. *N. Sativa* extract inhibited LPS-induced TNF-α and IL-6 expression and induced additive effect on LPS stimulated IL-8 and IL-10 expression. The exact mechanism by which NS extract induce its action is unclear. Ibrahim *et al.* (2008) showed that NS inhibited hepatotoxicity induced by carbon tetra chloride by down regulation of CYP2E and CYP3A protein expressions due to reduction of nitrous oxide through downregulation of inducible Nitrous Oxide Synthase (iNOS) expression. Salem (2005) reported that *N. sativa* inhibited the inflammatory cytokines IL-1 and TNF-α and enhanced IL-8 expression in human Peripheral Blood Mono-nuclear Cells (PBMC) through an alteration of trafficking of inflammatory cells via., modulating the expression of chemokines and or other adhesion molecules. Therefore, *N. sativa* helps to balance the inflammatory response during endotoxemia as reported by present findings and that of Howard *et al.* (1993).

IL-8 is a chemokine produced by a wide variety of cell types in response to LPS (Yoshimura *et al.*, 1987) by investigating the effect of *N. sativa* seed proteins on cytokine production in humans PBMC, it enhanced the production of IL-3 and IL-1 by lymphocytes when cultured with or without allogeneic cells (Haq *et al.*, 1995). Present findings showed the additive stimulatory effect of NS extract on LPS stimulated IL-1 expression and its role as an initiator of inflammation in cattle. Of note, stimulatory effect of whole *N. sativa* and their fractionated proteins was also noticed on the production of TNF-α by either non-activated or mitogen-activated PBMC (Haq *et al.*, 1999) and that are parallel to present results regardless the difference in cell types and species.

Interleukin-10 (IL-10) is a potent anti-inflammatory cytokine. It inhibits IL-1 and IL-6 production from macrophages (Fiorentino *et al.*, 1991). This cytokine plays a critical role in shaping

the immune response by blocking class II major histocompatibility complex expression and decreasing pro-inflammatory cytokine expression (Donnelly *et al.*, 1999; Moore *et al.*, 2001) and that is similar to present findings.

In conclusion, aqueous extract of NS showed immune-modulatory effect on bovine adipocytes through down-regulation of IL-6 and TNF and up-regulation of IL-10 and IL-8 expression induced by LPS treatment. Moreover, bovine adipocytes play an important role in balancing different inflammatory responses that happened during inflammation and or disease like blood mono-nuclear cells.

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